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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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Blatt 2 der Bescheinigung
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GENETICALLY ENGINEERED CELL LINES, AND THEIR USES, IN PARTICULAR FOR NEUROTOXICITY TESTING

The present invention relates to genetically engineered cell lines, and to their uses, in particular for testing the neurotoxicity of compounds, or for screening pharmaceutically active molecules, and for mechanistic studies.

Until now, a combination of whole animal models, and *in vitro* methodologies including freshly isolated neuronal cells or several neuronal cell lines, have been used for neurotoxicity screening (reviewed in Atterwil *et al.*, 1994). Apart from ethical considerations there are financial incentives for industries to reduce the use of animals in these studies. In recent years, *in vitro* technology using clonal cell lines has advanced so rapidly that it is now an accepted and valued option to solve toxicological problems. This development has also opened the way to the use of new sensitive *in vitro* endpoints, like apoptosis, potentially affected at realistic exposure .

Apoptosis is an active mode of cell death in contrast to necrosis, which is a process during which the cell expires by exhaustion. It is a fundamental physiological homeostatic process in multi-cellular organisms (Oltvai *et al.*, 1994) and significantly contributes to the organism defense against the persistence of cells with extensive DNA damage or mutations. Apoptosis is genetically controlled and influenced by extrinsic and intrinsic signals (Alison *et al.*, 1995) which may act to either suppress or promote the its activation (Steller, 1995).

Many drugs and environmental agents have been shown to induce apoptosis, even at concentrations insufficient to cause necrosis or general metabolic dysfunction. This is particularly the case in neurotoxicity where low levels of typical neurotoxic insults elicit apoptosis whereas high levels produce necrosis. The deregulation of apoptosis is involved in various chronic neuropathological diseases, including neuroblastomas, Alzheimer's, Parkinson's, HIV encephalopathy, Prion's and Huntington's, as well as in acute conditions such as stroke and traumatic injuries (reviewed by Bredesen, 1995). This has implications for both the pathophysiologies of neurodegenerative diseases and the development of therapeutic agents, as it may be possible to control apoptosis with the development of drugs that act against the molecular components of the death machinery (Steller, 1995). In fact,

several biotechnology companies are focusing on ways to control apoptosis with new therapeutics (Potera, 1998).

Apoptosis can provide information on exposure at low levels that do not result in disease symptoms and allows exposure assessments long before a chronic disease develops. Thus, being a very sensitive *in vitro* endpoint and at the same time having biological relevance, apoptosis can be considered as an ideal biomarker for xenobiotic exposure.

Expression of bcl-2 oncoprotein confers protection against apoptosis induced by many different types of stimuli (Hockenbery *et al.*, 1990, Yang *et al.*, 1997). On the other hand, the wild type (wt) p53 gene product is known to induce apoptosis (Yonish-Rouach *et al.*, 1991). Consequently, different expression levels of bcl-2 and wt p53 may affect cellular responses to chemicals, which may induce apoptosis. The development of genetically manipulated cells, displaying different sensitivities to apoptosis after introduction of wt p53 or bcl-2, can thus be a tool to understand the relevant molecular mechanisms involved in the development of cell death. Furthermore, these cell lines could be used to study the mechanisms for the activation or repression of cell death by compounds that interfere with the genetic machinery that controls apoptosis.

A rat pheochromocytoma cell line, PC12, which synthesizes and stores catecholamines, and responds to nerve growth factor protein (NGF) by extending long, branching neuronal-like processes (Greene and Tischler, 1976), has become increasingly popular in neurobiological research. This cell line constitutes a useful model for studying the mechanisms of apoptosis and its prevention (Sarafian *et al.*, 1994, Satoh *et al.*, 1996) or induction (Blum *et al.*, 1997).

In the present invention, the inventors achieved regulated, high level expression of wt p53 or bcl-2 in the neuronal cell line, PC12, by using a tetracycline regulated expression system (Gossen and Bujard, 1992). Here, the level of gene expressed can be closely regulated by varying the concentrations of tetracycline in the growth medium, tetracycline blocking the level of gene expressed. It therefore provides a «genetic switch» to regulate gene expression in a precise, reversible and quantitative way. This system allows the analysis of the same cell line expressing wt p53 or bcl-2 at different levels, rather than analyzing different cell clones, therefore eliminating the problem of clonal variation. Furthermore, during construction and maintenance of PC12 cell lines expressing wt p53 or

bcl-2 under tetracycline control, cells were grown in the presence of tetracycline, thus eliminating undesired effects due to long-term overexpression of these genes.

Thus, the inventors have established for the very first time cell lines transformed with p53 or bcl-2 coding sequences which can be used also for the detection of cell toxic compounds at very low concentrations, or for the detection of compounds having a pharmacological effect at very low concentrations.

The aim of the present invention is to provide new cell lines for carrying out processes for the detection of cell toxic compounds, or for the screening of drugs, or for mechanistic studies.

Another aim of the invention is to provide a new process for the detection of cell toxic compounds, i.e. compounds accelerating or inhibiting apoptosis in normal cells.

Another aim of the present invention is to provide a new process for the screening of drugs, either being capable of accelerating apoptosis in cells wherein apoptosis mechanism is abnormally inhibited, or being capable of inhibiting apoptosis in cells wherein apoptosis mechanism is abnormally enhanced.

Another aim of the present invention is to provide kits for carrying out said processes.

The invention relates to the use of cells genetically transformed with the following nucleotide sequences :

- a nucleotide sequence coding for the p53 protein or a fragment or a derived sequence thereof, said fragment or derived sequence being able to induce apoptosis,
- or a nucleotide sequence coding for the bcl-2 protein or a fragment or a derived sequence thereof, said fragment or derived sequence being able to inhibit apoptosis,
- and a nucleotide sequence controlling the level of expression of p53 or bcl-2 proteins, said nucleotide sequence comprising a sequence coding for an activator protein responsible for the expression of p53 or bcl-2 proteins mentioned above either when a specific compound is present in the culture medium, or when a specific compound is absent from the culture medium, the expression p53 or bcl-2 proteins ranging between 0% to 100% depending on the concentration of the specific compound in the culture medium, said specific compound being able to bind to said activator protein thus allowing, or conversely, inhibiting said protein to act as an activator of the promotion of the transcription under the

control of which is placed the above-mentioned nucleotide sequence coding for the p53 or bcl-2 protein,

for the detection of developmental and post-developmental toxicity of endogenous or exogenous factors, including drugs and other chemicals, and more particularly stress factors disrupting neuronal cell homeostasis, or for the screening of drugs for the treatment of pathologies related to an abnormal activation or inhibition of apoptosis, i.e. of drugs being capable of inhibiting cell death in cells wherein apoptosis is abnormally induced, or of drugs being capable of inducing cell death in cells wherein apoptosis is abnormally inhibited, respectively, or for mechanistic studies.

The invention relates more particularly to the above mentioned use of cells, characterized in that said genetically transformed cells are chosen among mammalian neuronal cells such as rat PC12 neuronal cell line or any other eucaryotic cells or cell lines.

The invention concerns more particularly the use of genetically transformed cells as mentioned above, for the detection of developmental and post-developmental toxicity of endogenous or exogenous factors, such as cell toxic compounds and more particularly neurotoxic compounds also at very low level exposure and neuronal death triggered by glutamate, reactive oxygen species, or hypoglycaemia.

The invention relates more particularly also to the use of genetically transformed cells as mentioned above, for the screening of drugs for the treatment of pathologies such as acute and chronic neurological disorders including stroke, and traumatic injuries, ageing, neuroblastomas, Alzheimer's disease, Parkinson's disease, HIV encephalopathy, Prion's disease, Creutzfeld-Jacob diseases, Huntington's disease and several neuromuscular diseases.

The invention also concerns more particularly the use of genetically transformed cells as mentioned above, for mechanistic studies, in particular :

- for identifying key mechanisms of neurodegeneration, i.e. processes at the molecular or cellular level which are critical for initiating and/or accelerating the neurodegenerative process,
- for studying in depth the involvement of overexpression of apoptotic genes (wt p53 and bcl-2) in protecting or destroying neuronal cells.

The transformed cells used according to the invention are more particularly characterized in that the expression of p53 or bcl-2 proteins is proportional to the quantity of the specific compound added to the culture medium, so that the level of expression of p53 or bcl-2 in the transformed cells is 0%, or 100%, or comprised between 0% and 100%.

The invention relates more particularly to the use of cells as described above, and characterized in that the nucleotide sequence controlling the level of expression of p53 or bcl-2 proteins, is a Tet-Off gene coding for a tetracycline-controllable transactivator (tTA), the tTA comprising a Tet repressor (TetR) linked to a polypeptide which directly or indirectly activates transcription of p53 or bcl-2 proteins, the transcription of the tTA being under the control of an appropriate promoter such as a promoter from cytomegalovirus (pCMV), and the specific compound added to the culture medium is tetracycline.

Advantageously, the above mentioned cells used according to the invention are characterized in that :

- the polypeptide which directly or indirectly activates transcription of p53 or bcl-2 proteins, is the virion protein 16 from herpes simplex virus (VP16),
- the transcription of the p53 or bcl-2 proteins is under the control of a tTA-responsive promoter comprising a minimal promoter, such as the cytomegalovirus immediate early gene promoter (P_{minCMV}), linked to a tetracycline responsive element (TRE).

The invention relates more particularly to the use of transformed cells as described above, characterized in that :

- in the presence of tetracycline at a concentration of at least approximately 0,01 µg/ml in the culture medium of transformed cells comprising approximately 150000 cells/ml, the level of expression of p53 or bcl-2 proteins in the transformed cells is considered as being 0%,
- in the absence of tetracycline, or for concentration of less than $2,5\ 10^{-4}$ µg/ml of tetracycline in the culture medium, the level of expression of p53 or bcl-2 proteins in the transformed cells is considered as being 100%, thus leading to transformed cells containing the p53 coding sequence which are more sensitive towards endogenous or exogenous triggers of cell death, when compared to said transformed cells wherein the level of expression of the p53 protein is 0%, and to transformed cells containing the bcl-2 coding

sequence which are more resistant towards endogenous or exogenous triggers of cell death, when compared to said transformed cells wherein the level of expression of the bcl-2 protein is 0%,

- the level of expression of p53 or bcl-2 proteins in the transformed cells ranging from 0% to 100% for concentrations of tetracycline ranging between approximately 0,01 µg/ml to concentration of less than 2,5 10^{-4} µg/ml, respectively.

The invention relates more particularly to the use of transformed cells as described above, for the detection of developmental and post-developmental toxicity of endogenous or exogenous factors, such as endogenous or exogenous stress factors disrupting neuronal cell homeostasis, as defined above, said detection being correlated :

a) in the transformed cells containing the p53 coding sequence, and expressing a specific proportion of the p53 protein (from 0% to 100%, depending on the quantity of the above-mentioned specific compound added to the culture medium of said cells, e.g. depending on for a given concentration of tetracycline), to an increase of the cell death threshold when compared to control transformed cells expressing the same specific proportion of the p53 protein and which have not been in contact with said toxic compounds,

b) in the transformed cells containing the bcl-2 coding sequence, and expressing a specific proportion of the bcl-2 protein (from 0% to 100%, depending on the quantity of the above-mentioned specific compound added to the culture medium of said cells, e.g. depending on for a given concentration of tetracycline), to a decrease of the cell death threshold when compared to control transformed cells expressing the same specific proportion of the bcl-2 protein and which have not been in contact with said toxic compounds.

Increased or decreased cell death threshold can be measured for example by cell viability assays such as MTT assay (Mosmann, 1983), or apoptosis assays such as morphological evaluations (Atterwill et al., 1994).

By way of illustration, the detection of a stress factor or cell toxic compound as defined above is correlated :

a) in the transformed cells containing the p53 coding sequence,

- and in the absence of tetracycline, to the observation of altered response to toxic insult, including increased cell death which can be measured by a method described above,

- and in the presence of increasing concentrations of tetracycline such that the expression of p53 decreases from 100% to 0%, to the observation of increased normalisation of the response to toxic insult which can be measured by a method described above,

b) in the transformed cells containing the bcl-2 coding sequence :

- and in the absence of tetracycline, to the observation of altered response to toxic insult, including decreased cell death which can be measured by a method described above,

- and in the presence of increasing concentrations of tetracycline such that the expression of bcl-2 decreases from 100% to 0%, to the observation of increased normalisation of the response to toxic insult which can be measured by a method described above.

Advantageously, endogenous or exogenous factors inducing toxicity, including drugs and other chemicals, can be detected in the frame of the present invention at very low concentrations using the above-mentioned cells containing the p53 coding sequence, i.e. at concentrations in the range of real world exposures, and more particularly in environmental (air, water, soil, radiation), food, cosmetic and drug samples.

The invention relates more particularly to the use of transformed cells as described above, for the screening of molecules useful as drugs in the treatment of pathologies related to an inhibition or an activation of apoptosis, such as pathologies listed above, the detection of said molecules being correlated :

a) in the transformed cells containing the p53 coding sequence, and expressing a specific proportion of the p53 protein, to a decrease of the cell death threshold when compared to control transformed cells expressing the same specific proportion of the p53 protein and which have not been in contact with the tested drug,

b) in the transformed cells containing the bcl-2 coding sequence, and expressing a specific proportion of the bcl-2 protein, to an increase of the cell death threshold when compared to control transformed cells expressing the same specific proportion of the bcl-2 protein and which have not been in contact with the tested drug.

Increased or decreased cell death threshold can be measured according to the methods described above.

By way of illustration, the detection of a drug as defined above is correlated :

a) in the transformed cells containing the p53 coding sequence,

- and in the absence of tetracycline, to the observation of a lowering of the cell death threshold which can be measured by a method described above,

- and in the presence of increasing concentrations of tetracycline such that the expression of p53 decreases from 100% to 0%, to the observation of a normalisation of the cell death threshold which can be measured by a method described above,

b) in the transformed cells containing the bcl-2 coding sequence :

- and in the absence of tetracycline, to the observation of an increase of the cell death threshold which can be measured by a method mentioned above,

- and in the presence of increasing concentrations of tetracycline such that the expression of bcl-2 decreases from 100% to 0%, to the observation of a normalisation of the cell death threshold which can be measured by a method mentioned above.

As a matter of fact, cells expressing increased levels of p53 mimics neuronal cells in patients with neurodegenerative diseases including cells being more sensitive towards cell death. Consequently, the possibility of increasing the sensitivity in this p53 cell line in a gradual way, allows said cell line to mimic perfectly acute and chronic neuronal disorders in which cells have varying degrees of increased sensitivity towards cell death such as HIV-encephalopathy. Cells expressing increased levels of bcl-2 mimics neuronal cells in patients with neurodegenerative diseases including cells being more resistant towards cell death. Consequently, the possibility of increasing the resistance in this bcl-2 cell line in a gradual way, allows said cell line to mimic perfectly chronic neuronal disorders in which cells have varying degrees of increased resistance towards cell death such as neuroblastomas.

The invention also relates to a process for the detection of developmental and post-developmental toxicity of endogenous or exogenous factors, including drugs and other chemicals, as defined above, or for the screening of molecules useful as drugs as defined above, said process comprising :

- contacting a sample comprising said compounds or molecules to be tested with one or several culture media of genetically transformed cells as described above wherein the expression of p53 or bcl-2 protein is from 0% to 100%, each culture medium containing cells expressing a specific proportion of p53 or bcl-2, said contacting being advantageously carried out at a temperature of approximately 37°C,

- the measure of the cell death threshold in the different cell expressing specific proportion of p53 or bcl-2 culture media, and the comparison of the measured threshold with the cell death threshold measured in control cells expressing the same specific proportion of p53 or bcl-2 proteins, said control cells having not been contacted with said sample.

Cell death thresholds in the above-mentioned process can be measured according to the methods described above, and the detection of a toxic compound or of a drug is correlated to an increase or a decrease of the cell death threshold as described above.

The invention also relates to the genetically transformed cells as described above, i.e. to the cells genetically transformed with the following nucleotide sequences :

- a nucleotide sequence coding for the p53 protein or a fragment or a derived sequence thereof, said fragment or derived sequence being able to induce apoptosis,

- or a nucleotide sequence coding for the bcl-2 protein or a fragment or a derived sequence thereof, said fragment or derived sequence being able to inhibit apoptosis,

- and a nucleotide sequence controlling the level of expression of p53 or bcl-2 proteins, said nucleotide sequence comprising a sequence coding for an activator protein responsible for the expression of p53 or bcl-2 proteins mentioned above either when a specific compound is present in the culture medium, or when a specific compound is absent from the culture medium, the expression p53 or bcl-2 proteins ranging between 0% to 100% depending on the concentration of the specific compound in the culture medium, said specific compound being able to bind to said activator protein thus allowing, or conversely, inhibiting said protein to act as an activator of the promotion of the transcription under the control of which is placed the above-mentioned nucleotide sequence coding for the p53 or bcl-2 protein.

The invention relates more particularly to the above mentioned genetically transformed cells chosen among mammalian neuronal cells such as rat PC12 neuronal cell line or any other eucaryotic cells or cell lines.

The invention relates more particularly to the cells as described above, and characterized in that the nucleotide sequence controlling the level of expression of p53 or bcl-2 proteins, is a Tet-Off gene coding for a tetracycline-controllable transactivator (tTA), the tTA comprising a Tet repressor (TetR) linked to a polypeptide which directly or indirectly activates transcription of p53 or bcl-2 proteins, the transcription of the tTA being under the control of an appropriate promoter such as a promoter from cytomegalovirus (pCMV).

Advantageously, the above mentioned cells used according to the invention are characterized in that :

- the polypeptide which directly or indirectly activates transcription of p53 or bcl-2 proteins, is the virion protein 16 from herpes simplex virus (VP16),

- the transcription of the p53 or bcl-2 proteins is under the control of a tTA-responsive promoter comprising a minimal promoter, such as the cytomegalovirus immediate early gene promoter (P_{minCMV}), linked to a tetracycline responsive element (TRE).

The invention also relates to kits for carrying out a process mentioned above for the detection of cell toxic compounds, such as endogenous or exogenous factors inducing cell death, as defined above, or for the screening of molecules useful as drugs as defined above, said kits comprising genetically transformed cells as described above according to the invention, and optionally the specific compound used to regulate the proportion of p53 or bcl-2 proteins in the culture medium, e.g. tetracycline, doxycycline.

The invention will be further illustrated with the following description of the preparation of the neuronal cell line PC12 transformed with the wt p53 or bcl-2 coding sequences and their uses for the study of the effects of camptothecin and inorganic mercury ($HgCl_2$).

I) MATERIALS AND METHODS

a) Materials

Cell culture dishes were from Corning Costar (Concorezzo, Italy). RPMI, penicillin G sodium, and streptomycin sulfate from Life Technologies (S. Giuliano Milanesi, Italy). Horse serum was from Biochrom (Berlin, Germany). Vitrogen 100 was from COLLAGEN (Ismaning, Germany). H-33342 and EH-1 were from Molecular Probes (Eugene, Oregon, USA). The vectors, pTet-Off, pTK-Hyg and pTRE, the PC12 Tet-Off cell line, fetal calf serum and hygromycin B were from GENZYME (Cinisello Balsamo, Italy). Enzymes were from Boehringer Mannhaim (Monza, Italy). The PVDF membrane and the BioRad DC protein assay kit II were from BioRad (Segrate, Italy). The ECL system was from Amersham (Milano, Italy). The human bcl-2 specific mouse mAb was from Santa Cruz (Heidelberg, Germany) and the human wt p53 specific mouse mAb from INALCO (Milano, Italy). All other reagents not further specified were from Sigma (Milano, Italy).

b) Plasmids

pTet-Off was originally described as PUHD15-1neo by Resnitzky et al. (1994) and was created by insertion of a neomycin resistance gene into PUHD15-1 (Gossen and Bujard, 1992). It expresses the tetracycline-responsive transcription activator, tTA, which is a tet-repressor-herpes simplex virus VP-16 fusion protein. pTRE was first described as pUHD10-3 by Gossen & Bujard (1992). It contains a heptmerised tet operator sequence, followed by cytomegalovirus minimal promoter, multiple cloning site, and the SV40 polyadenylation signal. In the absence of tetracycline molecules, tetracycline responsive transcription activators are able to bind to the tet operator sequences and activate transcription (Gossen and Bujard, 1992). To construct pTRE-bcl-2, pTRE and pSFFVneo-bcl-2 were digested with *Eco*R1, respectively, and purified by agarose gel electrophoresis to isolate the linearized pTRE and pSFFVneo-bcl-2 fragment containing the full coding sequence of bcl-2 cDNA. The resulting DNA fragments were ligated to generate pTRE-bcl-2, in which the bcl-2 cDNA was downstream of the tetracycline-responsive promoter. To construct pTRE-wt p53, pTRE was linearized with *Bam*H1, while pET3a-p53 with NdeI and BamH1. After removing the 5´overhang of the NdeI site the two DNA fragments were

ligated to form pTRE-wt p53, also containing the full wt p53 cDNA sequence downstream of the tetracycline-responsive promoter.

c) Cell culture and gene transfer

PC12 Tet-Off cell lines stably expressing the tetracycline-responsive transcription activator, were grown in RPMI supplemented with 10 % (v/v) horse serum, 5 % (v/v) fetal calf serum, 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, 150 µg/ml geneticin at 37 °C in a humidified incubator with 5 % CO_2, on Vitrogen 100 coated cell culture dishes. For the introduction of wt p53 or bcl-2 cDNAs into PC12 Tet-Off cells, 40 µg of circular pTRE-wt p53 or pTRE-bcl-2 were electroporated together with 2 µg of circular pTK-Hyg, containing the hygromycin resistance gene, into PC12 Tet-Off cells by using the Bio-Rad Gene pulser system. The electroporation was performed at 250 V and 960 µF in electroporation buffer (Parker and Stark, 1979) containing 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM D-Glucose, 21 mM Hepes (pH 7.1). Clones resistant to 150 µg/ml hygromycin B were tested for tetracycline-controlled expression of bcl-2 or wt p53 by Western Blot analysis.

d) Western Blot analysis

Cell lysis was performed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02 % sodium azide, 0.1 % SDS, 100 µg/ml PMSF (phenylmethylsulfonyl fluride), 1 µg/ml aprotinin, 1 % NP-40 (Nonidet P-40) and 0.5 % sodium deoxycholate. Quantification of protein was performed by using the BioRad DC protein assay kit II. 50 µg total protein was loaded in each lane onto a 12 % SDS-PAGE and transferred to PVDF (polyvinylidene difluride) membrane by using the BioRad transfer apparatus. The filters were probed by using human bcl-2 specific or human wt p53 specific mouse mAbs. The antibodies were used at a concentration of 0.1 µg/ml and detected by using the enhanced chemoluminescence (ECL) system. Densities of wt p53 or bcl-2 bands in Western Blots were scanned with a hp ScanJet 4C and the densitometric evaluation was performed by using the NIH Image 1.61 software.

e) Viability assays

To quantify apoptosis and secondary lysis, cells were loaded with a combination of the red fluorescent, membrane impermeable dye ethidium homodimer-1 (EH-1) (1 μM) and the blue fluorescent, membrane permeable dye H-33342 (0.5 μg/ml) for 5 min. The viability of cells was evaluated by monitoring simultaneously plasma membrane integrity (EH-1-exclusion) and morphological changes of the nucleus (H-33342 staining). Live cells displayed intact nuclei and plasma membrane. Apoptotic cells showed intact plasma membrane with highly hyperfluorescent and condensed nuclei. In necrotic cells the plasma membrane was damaged and in their intact nuclei the EH-1 stain displaced the fainter H-33342 stain. Cell counts were performed on three 24 well dishes per cell line and condition and six randomly chosen optical fields per well with a Leica DM IRBE at 400 x magnification with one fluorescent filter setting. Each experiment was repeated at least three times. In addition, the percentage of viable cells was quantified by their MTT-reducing capacity after incubation with 0.5 mg/ml MTT for 2 h (Mosmann, 1983). The medium was then removed, lysing buffer containing 95 % ispropanol and 5 % formic acid was added, and the absorbance was detected at λ=570 nm and λ= 630 nm on a multiscan reader. Individual experiments were run as triplicates. Each of these experiments was repeated at least three times.

f) Differentiation of PC12 Tet-Off cells expressing wt p53 or bcl-2 under tetracycline control

Cells were exposed to growth medium, in the presence or absence of 2 μg/ml tetracycline for 2 or 8 days. Thereafter, they were exposed to 50 ng/ml 7 S NGF in serum reduced growth medium, containing 1 % horse serum, in the presence or absence of 2 μg/ml tetracycline. Changes in cell morphology were monitored in a 24 h rhythm for 5 days. The ability of cells to respond to NGF stimulation was evaluated by monitoring the appearance of neurite-like outgrowths. Cell counts were performed on 3 wells per cell line and 6 randomly chosen optical fields per 12 well dish with a Leica DM IRBE at 200 x magnification by using phase contrast microscopy. Each experiment was repeated at least three times.

II) RESULTS

a) Construction of PC12 Tet-Off cell lines expressing wt p53 or bcl-2 under tetracycline control.

Using the tetracycline-controlled expression system, 8 independent hygromycin resistant clones from pTRE-wt p53 and 4 independent hygromycin resistant clones from pTRE-bcl-2 transfected PC12 Tet-Off cells were examined at the protein level. 5 of the 8 pTRE-wtp53 transfected clones and 3 out of 4 analyzed pTRE-bcl-2 transfected clones showed high, inducible wt p53 or bcl-2 expression. In these clones, wt p53 and bcl-2 were strongly expressed in the absence of tetracycline, whereas in the presence of 2 µg/ml tetracycline both proteins were undetectable by Western Blot analysis. One PC12 Tet-Off cell line expressing wt p53 and one expressing bcl-2 in a regulated fashion were selected for the experiments discussed in the present described work. To quantitatively control wt p53 or bcl-2 expression, the PC12 cell lines expressing wt p53 or bcl-2 under tetracycline control were grown in eight different concentrations of this antibiotic. Western Blot analysis shows that tetracycline quantitatively controls gene expression in both cell lines (Fig.1).

b) Kinetics of wt p53 or bcl-2 expression in double stable PC12 Tet-Off cell lines.

The kinetics of wt p53 or bcl-2 induction was examined over a period of seven days in PC12 cell lines expressing wt p53 or bcl-2 under tetracycline control, by using Western Blot analysis. In both cell lines steady-state protein levels are reached 24 h after induction (removal of tetracycline from the growth medium) and there is very little fluctuation in the levels of bcl-2 or wt p53 expression during the rest of the induction period (Fig.2). In experiments described in the present described work, cells were grown in the appropriate tetracycline concentration for at least 48 h before the assay to ensure the maximal effect of tetracycline on bcl-2 or wt p53 expression.

c) Bcl-2 enhances induction of differentiation by NGF in PC12 cell lines expressing bcl-2 under tetracycline control.

PC12 cells cultured in serum containing medium without NGF resemble normal chromaffin cells, while NGF causes them to cease dividing and to develop neurite-like processes (Greene and Tischler, 1976; Tischler and Greene 1978). To verify if wt p53 or bcl-2 expressing PC12 Tet-Off cell lines retained their differentiation potential both cell lines were grown in the presence or absence of 2 μg/ml tetracycline for 2 days (induction period) and thereafter exposed to NGF for six days, again in the presence or absence of 2 μg/ml tetracycline. In PC12 cell lines expressing wt p53 or bcl-2 under tetracycline control, a high percentage of cells generated neurite-like outgrowths after a 5 day NGF treatment, which was comparable between cells grown in the presence or absence of tetracycline (Fig. 3a, data for PC12 cell lines expressing wt p53 under tetracycline control are not shown). No differences in the percentages of cells with neurite-like processes could be detected between cells grown in the presence or absence of tetracycline at any timepoint of NGF treatment. Katoh *et al.* (1996) reported that NGF induces the level of bcl-2 and that bcl-2 antisense oliogonucleotide inhibits neuronal differentiation of PC12 cells induced by NGF, suggesting that bcl-2 is involved in NGF induced neuronal differentiation. On the other hand, Batistatou *et al.* (1993) reported that bcl-2 expression did not affect neuronal differentiation in PC12 cells. In these works non-inducible expression systems were used to express bcl-2, leaving the possibility of pleiotropic effects due to permanent overexpression of bcl-2. We investigated whether bcl-2 expression might interfere with the differentiating actions of NGF by using PC12 cells expressing bcl-2 under tight tetracycline control. To examine this issue the induction period was prolonged from 48 h to 8 days. Under these conditions, a significant difference was detected in the percentage of differentiated cells between cells grown in the absence or presence of gene induction, 24 and 48 h after addition of NGF (Fig. 3b), suggesting the influence of bcl-2 in the differentiation process. Furthermore, both wt p53 and bcl-2 expression influenced the growth parameters of PC12 Tet-Off cells, indicating the importance of expressing genes of interest under tight control, as these might interfere with various processes in the genetically engineered cell line cell line during its development and maintenance.

d) Bcl-2 expression protects from cell death induced by $HgCl_2$ or camptothecin, while wt p53 expression potentiated cell death induces by these compounds

The effects of bcl-2 or wt p53 expression on cell death induced by camptothecin and $HgCl_2$, were established in wt p53 and bcl-2 expressing PC12 Tet-Off cell lines grown in the presence (control) or absence of tetracycline. Camptothecin induced neurotoxicity has been attributed to transcriptionally mediated DNA strand break formation (Morris and Geller, 1996) and consequent cell cycle signaling components (Park *et al.*, 1997). $HgCl_2$ is a well-known neurotoxic compound, which can cause alterations in the cellular response to NGF induced differentiation or result in cell death by facilitating Ca^{2+} entry through membrane channels (Rossi *et al.*, 1993, 1997). To assess bcl-2 or wt p53 effects on camptothecin or $HgCl_2$ induced cell death, we monitored the viability of cells by morphological evaluations (Fig. 4 A, C) giving the possibility to distinguish between apoptotic and necrotic cells and by their capacity to sequester and metabolize MTT (Fig. 4 B, D). When bcl-2 expression was induced in PC12 Tet-Off cells, cell death, assessed by morphological evaluations, was delayed in response to both camptothecin and $HgCl_2$. On the other hand, wt p53 expression enhanced cell death in PC12 Tet-Off cells induced by these compounds (Fig. 4 A, C). The mechanism by which camptothecin or $HgCl_2$ induced cell death in PC12 Tet-Off cell lines, expressing wt p53 or bcl-2 under tetracycline control, was apoptosis rather than necrosis. In camptothecin treated PC12 Tet-Off cells, mitochondrial MTT metabolism was increased by bcl-2 and decreased by wt p53 expression (Fig.4 D). On the other hand, no significant differences could be detected in MTT metabolism between control (PC12 Tet-Off cell lines in which wt p53 or bcl-2 expression is blocked by tetracycline), wt p53 and bcl-2 expressing PC12 Tet-Off cell lines (Fig. 4B) exposed to $HgCl_2$. Thus, the MTT assay was sensitive enough for picking up differences in sensitivities towards cell death between control, wt p53 and bcl-2 expressing PC12 Tet-Off cell lines exposed to camptothecin, but failed to do so in $HgCl_2$ treated cells. This suggests that the morphological evaluation, giving the possibility to detect also apoptotic cells, may be a more sensitive endpoint of exposure compared to the MTT reducing capacity of the cell.

III) CONCLUSION

Although a single cell line does not reflect the complexity of the nervous system, primary neurons or neuronal cell lines are frequently used for mechanistic studies or for pre-screening purposes. The PC12 cell line is a good and highly used *in vitro* model in neurobiological sciences. Here, genetically engineered neuronal PC12 cell lines were constructed, expressing wt p53 or bcl-2, the key players of the machinery which controls apoptosis, under tight, reversible and quantitative control of tetracycline, which in this system blocks the level of gene expressed. Thereafter, it has been tested whether PC12 Tet-Off cell lines expressing wt p53 or bcl-2 under Tet-Off control, may provide cells with different sensitivities to apoptosis, after growing them in the presence or absence of tetracycline and thereafter exposing them to the neurotoxic compound, $HgCl_2$, or the DNA damaging agent camptothecin. The morphological evaluation of viability showed that bcl-2 expressing PC12 Tet-Off cells are significantly more resistant to apoptosis induced by camptothecin or $HgCl_2$, while wt p53 expressing cells are significantly more sensitive than PC12 Tet-Off cells grown in the absence of gene expression. Therefore, after addition of different concentrations of tetracycline to the growth medium, these two cell lines provide a panel of cells with different sensitivities towards apoptosis. In addition it was shown that PC12 Tet-Off cell lines expressing wt p53 or bcl-2 under tetracycline control, have both retained their differentiation power in response to NGF stimulation, giving the possibility to use differentiation, besides apoptosis or necrosis as endpoint of exposure. Furthermore, it was shown that bcl-2 enhances the development of neurite-like processes of PC12 Tet-Off cells after addition of NGF and that both wt p53 and bcl-2 influence the growth parameters of PC12 Tet-Off cells. Thus, the advantages of such a testing system include 1) the possibility of modulating the sensitivity of PC12 cells, without moderating the growth parameters 2) the possibility to discriminate between genotoxic and cytotoxic compounds 3) the possibility to differentiate PC12 cells in the absence of gene expression, thereafter growing them in presence or absence of expression of wt p53 or bcl-2 and exposing them to toxic compounds 4) the possibility of evaluating also nervous system specific endpoints such as differentiation and ion channel permeability due to the origin of the cell line.

To conclude, a non-complex neurotoxicity model has been developed, based on a single neuronal cell lines, engineered to provide a panel of cells with different sensitivities towards cell death along to their specific neuronal cell characteristics.

In addition to being used for mechanistic studies, they can be used for testing developmental and postdevelopmental neurotoxicity of drugs and other chemicals, also at low-level exposure, as well as for the testing of neuroprotective drugs being capable of inhibiting cell death in cells wherein apoptosis is abnormally induced, or of drugs being capable of inducing cell death in cells wherein apoptosis is abnormally inhibited.

FIGURE LEGENDS

FIG. 1 Quantitative control of wt p53 or bcl-2 expression in PC12 Tet-Off cell lines. PC12 cell lines expressing wt p53 or bcl-2 under tetracycline control were cultured for 48 hours in the indicated concentrations of tetracycline (Tc) and analyzed by Western Blotting as detailed in Materials and Methods. Densities of wt p53 or bcl-2 bands in Western Blots were scanned with a hp ScanJet 4C/T, quantified by using the NIH Image 1.61 software and thereafter plotted against tetracycline concentrations.

FIG. 2 Kinetics of wt p53 or bcl-2 expression in PC12 Tet-Off cell lines. PC12 Tet-Off cell lines transfected with pTRE-wt p53 or pTRE-bcl-2 were grown in the presence or absence of 2 μg/ml tetracycline (Tc) and analyzed by Western Blotting as described in Materials and Methods.

FIG. 3 Bcl-2-expression enhances development of neurite outgrowths in response to NGF stimulation, in PC12 Tet-Off cell lines. PC12 cells expressing bcl-2 in a regulated fashion were grown in the presence or absence of 2 μg/ml tetracycline for 48 hours (A) or 8 days (B). Thereafter, they were exposed to 50 ng/ml NGF for the indicated period of time, in the presence (open squares) or absence (closed squares) of 2 μg/ml tetracycline. Triplicate cultures were scored for neurite-bearing cells. Data shown are mean ± SD (n=3).

FIG. 4 Inhibition of cell death by bcl-2 and induction by wt p53 in double stable PC12 Tet-Off cell lines. PC12 cell lines expressing bcl-2 (squares) or wt p53 (triangles) under tetracycline control were grown in the presence (open symbols) or absence (closed symbols) of 2 μg/ml tetracycline for 48 hours before treatment. During (C, D) or after (A, B) treatments cells were grown again in the presence or absence of 2 μg/ml tetracycline. In A and B cells were treated for 10 minutes with the indicated concentrations of $HgCl_2$ and the percentage of vital cells determined 24 hours after beginning of the treatment; in C and D the percentage of vital cells was determined after 24 hours exposure to the indicated concentrations of camptothecin. In A and C, cells were double stained with ethidium homodimer (EH-1) and H-33342 and thereafter scored for vital cells; in B and D the vitality of cells was determined by using the MTT-assay. Data shown are mean ± s.e.m. of three independent experiments performed in triplicate.

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CLAIMS

1. Use of cells genetically transformed with the following nucleotide sequences :

- a nucleotide sequence coding for the p53 protein or a fragment or a derived sequence thereof, said fragment or derived sequence being able to induce apoptosis,

- or a nucleotide sequence coding for the bcl-2 protein or a fragment or a derived sequence thereof, said fragment or derived sequence being able to inhibit apoptosis,

- and a nucleotide sequence controlling the level of expression of p53 or bcl-2 proteins, said nucleotide sequence comprising a sequence coding for an activator protein responsible for the expression of p53 or bcl-2 proteins mentioned above either when a specific compound is present in the culture medium, or when a specific compound is absent from the culture medium, the expression p53 or bcl-2 proteins ranging between 0% to 100% depending on the concentration of the specific compound in the culture medium, said specific compound being able to bind to said activator protein thus allowing, or conversely, inhibiting said protein to act as an activator of the promotion of the transcription under the control of which is placed the above-mentioned nucleotide sequence coding for the p53 or bcl-2 protein,

for the detection of developmental or post-developmental toxicity of endogenous or exogenous factors, including drugs and other chemicals, or for the screening of drugs for the treatment of pathologies related to an abnormal inhibition or activation of apoptosis, or for mechanistic studies.

2. Use according to claim 1, characterized in that the genetically transformed cells are chosen among mammalian neuronal cells such as rat PC12 neuronal cell line or any other eucaryotic cells or cell lines.

3. Use according to claim 1 or 2, characterized in that the expression of p53 or bcl-2 proteins is proportional to the quantity of the specific compound added to the culture medium, so that the level of expression of p53 or Bcl-2 in the transformed cells is 0%, or 100%, or comprised between 0% and 100%.

4. Use according to anyone of claims 1 to 3, characterized in that the nucleotide sequence controlling the level of expression of p53 or bcl-2 proteins, is a Tet-Off gene coding for a tetracycline-controllable transactivator (tTA), the tTA comprising a Tet repressor (TetR) linked to a polypeptide which directly or indirectly activates transcription of p53 or Bcl-2 proteins, the transcription of the tTA being under the control of an appropriate promoter such as a promoter from cytomegalovirus (pCMV).

5. Use according to anyone of claims 1 to 4, characterized in that :

- the polypeptide which directly or indirectly activates transcription of p53 or bcl-2 proteins, is the virion protein 16 from herpes simplex virus (VP16),
- the transcription of the p53 or bcl-2 proteins is under the control of a tTA-responsive promoter comprising a minimal promoter, such as the cytomegalovirus immediate early gene promoter (P_{minCMV}), linked to a tetracycline responsive element (TRE).

6. Cells genetically transformed with the following nucleotide sequences :

- a nucleotide sequence coding for the p53 protein or a fragment or a derived sequence thereof, said fragment or derived sequence being able to induce apoptosis,
- or a nucleotide sequence coding for the bcl-2 protein or a fragment or a derived sequence thereof, said fragment or derived sequence being able to inhibit apoptosis,
- and a nucleotide sequence controlling the level of expression of p53 or bcl-2 proteins, said nucleotide sequence comprising a sequence coding for an activator protein responsible for the expression of p53 or bcl-2 proteins mentioned above either when a specific compound is present in the culture medium, or when a specific compound is absent from the culture medium, the expression p53 or bcl-2 proteins ranging between 0% to 100% depending on the concentration of the specific compound in the culture medium, said specific compound being able to bind to said activator protein thus allowing, or conversely, inhibiting said protein to act as an activator of the promotion of the transcription under the control of which is placed the above-mentioned nucleotide sequence coding for the p53 or bcl-2 protein.

7. Genetically transformed cells according to claim 6, characterized in that they are chosen among mammalian neuronal cells such as rat PC12 neuronal cell line or any other eucaryotic cells or cell lines.

8. Genetically transformed cells according to claim 6 or 7, characterized in that the nucleotide sequence controlling the level of expression of p53 or bcl-2 proteins, is a Tet-Off gene coding for a tetracycline-controllable transactivator (tTA), the tTA comprising a Tet repressor (TetR) linked to a polypeptide which directly or indirectly activates transcription of p53 or bcl-2 proteins, the transcription of the tTA being under the control of an appropriate promoter such as a promoter from cytomegalovirus (pCMV).

9. Genetically transformed cells according to anyone of claims 6 to 8, characterized in that :

- the polypeptide which directly or indirectly activates transcription of p53 or bcl-2 proteins, is the virion protein 16 from herpes simplex virus (VP16),
- the transcription of the p53 or bcl-2 proteins is under the control of a tTA-responsive promoter comprising a minimal promoter, such as the cytomegalovirus immediate early gene promoter (P_{minCMV}), linked to a tetracycline responsive element (TRE).

10. Process for the detection of developmental and post-developmental toxicity of endogenous or exogenous factors, including drugs and other chemicals, or for the screening of molecules useful as drugs for the treatment of pathologies related to an inhibition or an activation of apoptosis, said process comprising :

- contacting a sample comprising said compounds or molecules to be tested with one or several culture media of genetically transformed cells as defined in anyone of claims 6 to 9, wherein the expression of p53 or bcl-2 protein is from 0% to 100%, each culture medium containing cells expressing a specific proportion of p53 or bcl-2,
- the measure of the cell death threshold in the different cell expressing specific proportion of p53 or bcl-2 culture media, and the comparison of the measured threshold

with the cell death threshold measured in control cells expressing the same specific proportion of p53 or bcl-2 proteins, said control cells having not been contacted with said sample.

11. Process according to claim 10, for the screening of drugs for the treatment of pathologies such as acute and chronic neurological disorders including stroke, and traumatic injuries, ageing, neuroblastomas, Alzheimer's disease, Parkinson's disease, HIV encephalopathy, Prion's disease, Creutzfeld-Jacob diseases, Huntington's disease and several neuromuscular diseases.

12. Kits for carrying out a process according to claim 10 or 11, comprising genetically transformed cells according to anyone of claims 6 to 9, and optionally the specific compound used to regulate the proportion of p53 or bcl-2 proteins in the culture medium, such as tetracycline or doxycycline.

ABSTRACT

GENETICALLY ENGINEERED CELL LINES, AND THEIR USES, IN PARTICULAR FOR NEUROTOXICITY TESTING

The present invention relates to genetically engineered cell lines, and to their uses, in particular in processes for screening the developmental or post-developmental neurotoxicity of endogenous or exogenous factors, including drugs and other chemicals, also at low-level exposure, for screening pharmaceutically active molecules useful as drugs for the treatment of pathologies involving an abnormal inhibition or activation of cell death, and for mechanistic studies, the said cell lines being genetically transformed with a nucleotide sequence coding for the p53 protein or a nucleotide sequence coding for the bcl-2 protein, and a nucleotide sequence controlling the level of expression of p53 or bcl-2 proteins. The present invention also relates to kits comprising the said genetically engineered cell lines for carrying out the said processes.

(no figure)

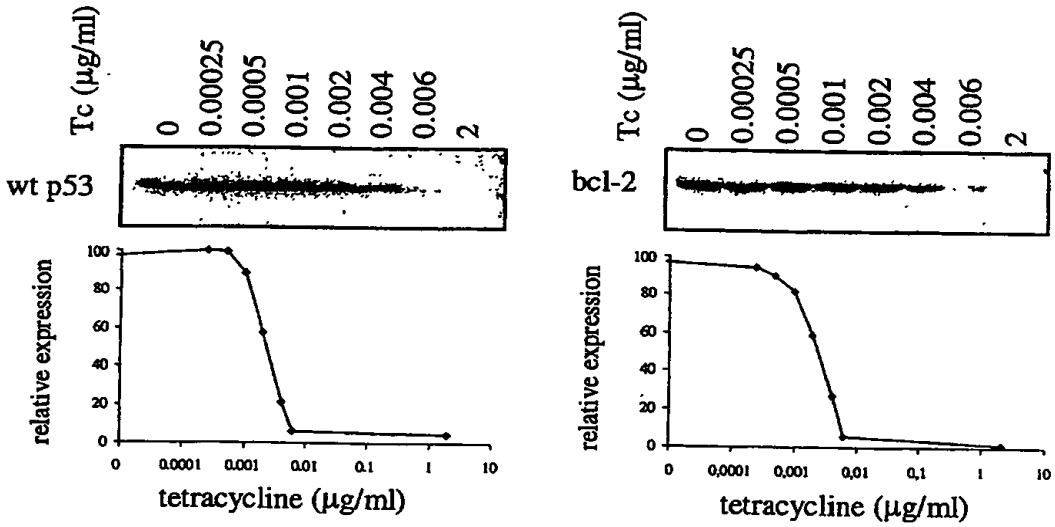
Tc (µg/ml)
0
0.00025
0.0005
0.001
0.002
0.004
0.006
2
wt p53
bcl-2
relative expression
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
10
tetracycline (µg/ml)

Figure 1

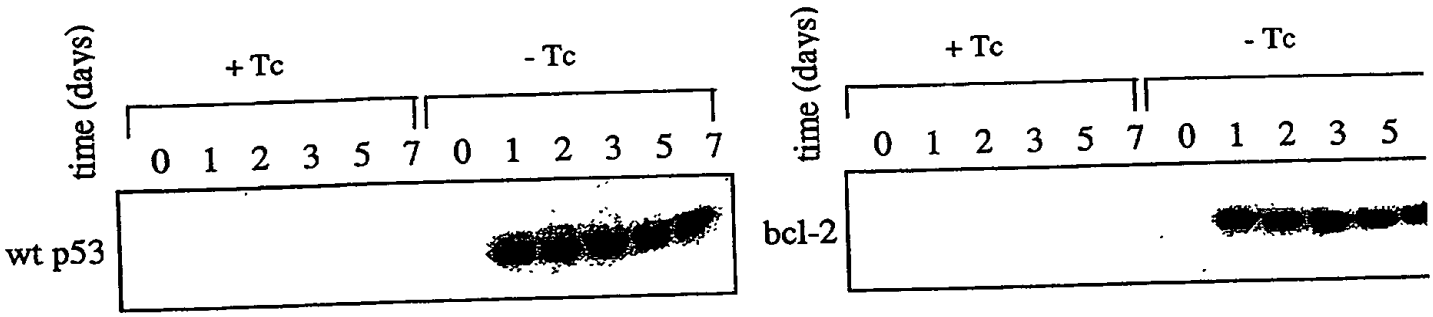
time (days)
+ Tc
- Tc
0 1 2 3 5 7 0 1 2 3 5 7
wt p53
time (days)
+ Tc
- Tc
0 1 2 3 5 7 0 1 2 3 5
bcl-2

Figure 2

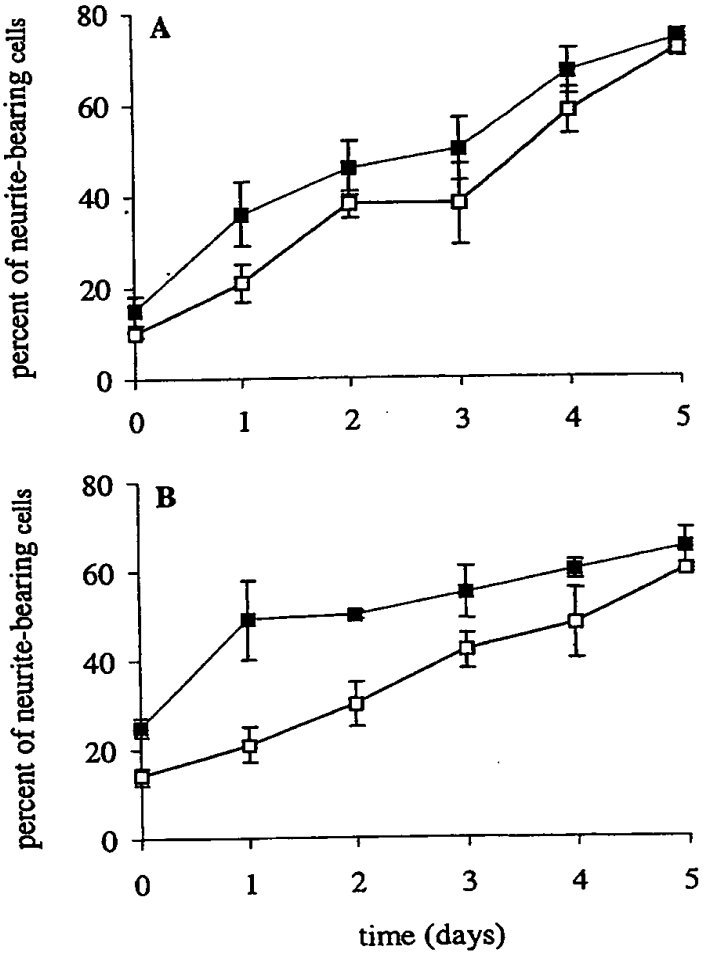
A
B
percent of neurite-bearing cells
percent of neurite-bearing cells
80
60
40
20
0
0
1
2
3
4
5
time (days)

Figure 3

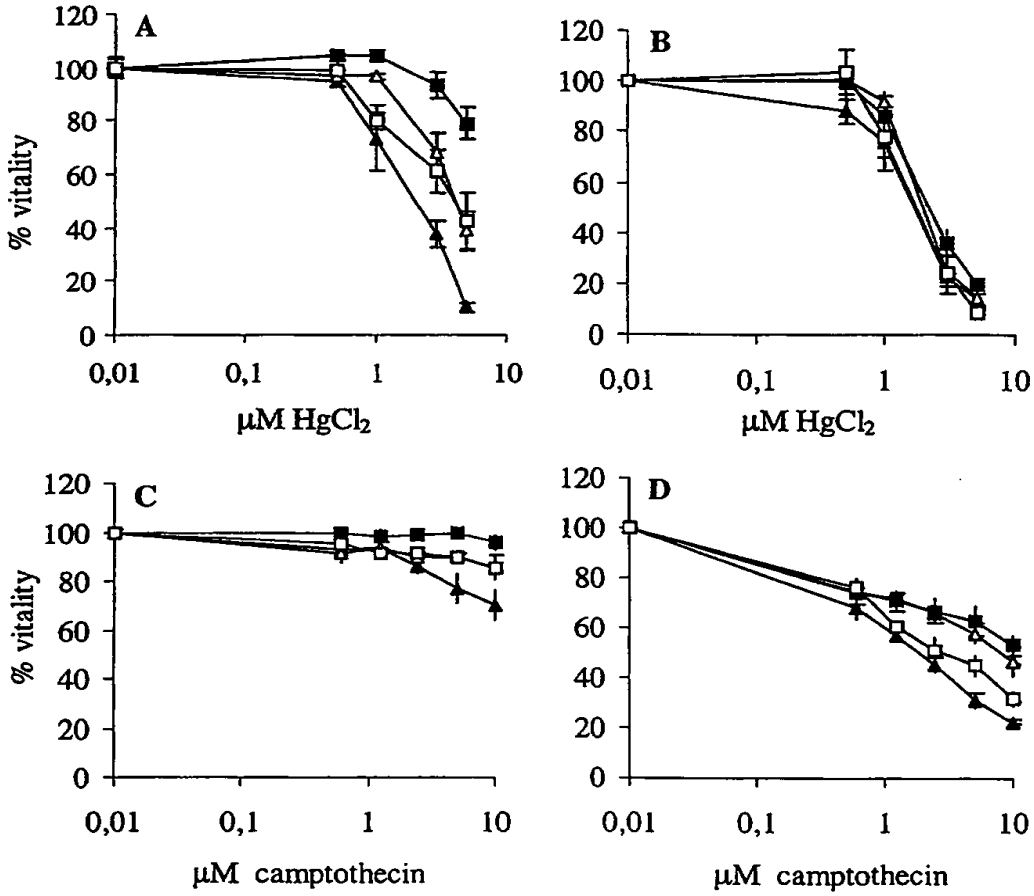

Figure 4